

FORM PTO-1390
(REV 10-95)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

**TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. §371**

LEVER 620X (F 7371 (C))

U.S. APPLICATION NO. (If known, see 37 CFR §1.5)

09/308140

INTERNATIONAL APPLICATION NO.

PCT/EP97/06181

INTERNATIONAL FILING DATE

06 November 1997

PRIORITY DATE CLAIMED

19 November 1996

TITLE OF INVENTION

CARROT ANTIFREEZE POLYPEPTIDES

APPLICANT(S) FOR DO/EO/US

Louise Jane BYASS, et al

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. §371.
 2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. §371.
 3. ☒ This express request to begin national examination procedures (35 U.S.C. §371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. §371(b) and PCT Articles 22 and 39(1).
 4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
 5. ☒ A copy of the International Application as filed (35 U.S.C. §371(c)(2))
 - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☒ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
 6. ☒ A translation of the International Application into English (35 U.S.C. §371(c)(2)). **FILED IN ENGLISH**
 7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. §371(c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
 8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. §371(c)(3)).
 9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. §371(c)(4)).
 10. ☒ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. §371(c)(5)).
- Items 11. to 16. below concern document(s) or information included:**
11. ☐ An Information Disclosure Statement under 37 C.F.R. §§1.97 and 1.98.
 12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. §§3.28 and 3.31 is included.
 13. ☒ A FIRST preliminary amendment.
 - ☐ A SECOND or SUBSEQUENT preliminary amendment.
 14. ☐ A substitute specification.
 15. ☐ A change of power of attorney and/or address letter.
 16. ☒ Other items or information:

A Submission of Article 34 Amendments with amended pages 39-42 is also being filed concurrently herewith.

U.S. APPLICATION NO. (if known, see 37 CFR §1.5)		INTERNATIONAL APPLICATION NO. PCT/EP97/06181		ATTORNEY'S DOCKET NUMBER LEVER 620X (F 7371(C))	
17. <input checked="" type="checkbox"/> The following fees are submitted:				CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR §1.492 (a) (1) - (5)):					
Search Report has been prepared by the EPO or JPO.....				\$840.00	
International preliminary examination fee paid to USPTO (37 CFR §1.482).....				\$670.00	
No international preliminary examination fee paid to USPTO (37 CFR §1.482) but international search fee paid to USPTO (37 CFR §1.445(a)(2)).....				\$760.00	
Neither international preliminary examination fee (37 CFR §1.482) nor international search fee (37 CFR §1.445(a)(2)) paid to USPTO.....				\$970.00	
International preliminary examination fee paid to USPTO (37 CFR §1.482) and all claims satisfied provisions of PCT Article 33(2)-(4).....				\$96.00	
ENTER APPROPRIATE BASIC FEE AMOUNT =				\$840.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than months from the earliest claimed priority date (37 C.F.R. §1.492(e)). <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30				\$130.00	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	20 - 20 =	0	x \$ 18.00	\$0.00	
Independent claims	1 - 3 =	0	x \$ 78.00	\$0.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)				+ \$ 260.00	
TOTAL OF ABOVE CALCULATIONS =				\$970.00	
Reduction of 1/2 for filing by small entity, if applicable. A Verified Small Entity Statement must also be filed (Note 37 C.F.R. §§1.9, 1.27, 1.28).					
SUBTOTAL =				\$970.00	
Processing fee of \$130.00 for furnishing the English translation later than months from the earliest claimed priority date (37 C.F.R. §1.492(f)). <input type="checkbox"/> 20 <input type="checkbox"/> 30					
TOTAL NATIONAL FEE =				\$970.00	
Fee for recording the enclosed assignment (37 C.F.R. §1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. §§3.28, 3.31). \$40.00 per property.					
TOTAL FEES ENCLOSED =				\$970.00	
				Amount to be refunded:	
				charged:	

- a. ☐ A check in the amount of \$0.00 to cover the above fees is enclosed.
- b. ☒ Please charge my Deposit Account No. 12-1155 in the amount of \$ 970.00 to cover the above fees. A duplicate copy of this sheet is enclosed.
- c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 12-1155. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 C.F.R. §§1.494 or 1.495 has not been met, a petition to revive (37 C.F.R. §1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

UNILEVER UNITED STATES
c/o MRS. LINDA HORVATH
Patent Department
45 River Road
Edgewater, New Jersey 07020

Anthony J. Zelano
SIGNATURE *30/5/97*

Anthony J. Zelano
NAME

Filed: 14 May 1999

AJZ:jvbp

27,969
REGISTRATION NUMBER

IN THE UNITED STATES DESIGNATED/ELECTED OFFICE

International Application No. : PCT/EP97/06181
International Filing Date : 06 November 1997
Priority Date Claimed : 19 November 1996
Applicant(s) (DO/EO/US) : BYASS, Louise Jane et al.

Title: CARROT ANTIFREEZE POLYPEPTIDES

PRELIMINARY AMENDMENT

BOX PCT
Assistant Commissioner for Patents
Washington, D.C. 20231

SIR:

Please amend the above-identified International application as follows prior to calculation of the fees.

IN THE CLAIMS:

Please **amend claims 5, 7, 8, 10, 12, 14 and 15** as follows:

Claim 5, line 2: Change "one or more of Claims 1- 4" to -- Claim 1 --.

Claim 7, lines 1 and 2: Change "one or more of Claims 1- 4" to -- Claim 1 --.

Claim 8, lines 1 and 2: Change "one or more of Claims 1- 4" to -- Claim 1 --.

Claim 10, line 2: Change "Claims 1, 2, 3 or 4" to -- Claim 1 --.

Claim 12, lines 1 and 2: Change "Claims 1, 2, 3 or 11" to --Claim 1--.

Claim 14, lines 2 and 3: Change "one or more claims 1, 2, 3 or 4" to -- Claim 1 --.

Claim 15, line 1: Change "Claims 1, 2, 3 or 4" to -- Claim 1 --.

Please add claims 17 through 20:

17. An antibody capable of specifically binding the polypeptide of claim 2.
18. A polypeptide that is immunologically related to the polypeptide of claim 2 as determined by its cross reactivity with an antibody capable of specifically binding the polypeptide of claim 2.
19. Food product comprising a polypeptide of claim 2 with the proviso that the food product is not a carrot containing the polypeptide at naturally occurring levels.

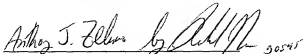
20. Method of producing a food product comprising an antifreeze polypeptide according to claim 2, comprising the steps of

- (a) adding to the food product a composition
comprising said antifreeze polypeptide; or
- (b) in situ production of said antifreeze polypeptide.

REMARKS

The principal purpose of this Preliminary Amendment is to eliminate multiple dependencies in order to avoid extra fees.

Respectfully submitted,

 56545

Anthony J. Zelano (Reg. No. 27,969)
Attorney for Applicants

MILLEN, WHITE, ZELANO & BRANIGAN, P.C.
Arlington Courthouse Plaza I
2200 Clarendon Boulevard, Suite 1400
Arlington, Virginia 22201
Direct Dial: 703-812-5311
Facsimile: 703-243-6410
Internet Address: zelanoa@mwzb.com

Filed: 14 May 1999

CARROT ANTIFREEZE POLYPEPTIDESTechnical Field of the Invention

- 5 The invention relates to anti-freeze polypeptides (AFPs) and food product containing AFPs.

Background to the Invention

- 10 Anti-freeze polypeptides (AFPs) have been suggested for improving the freezing tolerance of foodstuffs.

For the purpose of the invention, the term AFP has the meaning as well-known in the art, namely those proteins which
15 inhibit the growth of ice-crystals. See for example US 5,118,792.

- WO 90/13571 discloses antifreeze peptides produced chemically or by recombinant DNA techniques from plants. The AFPs can
20 suitably be used in food-products such as ice-cream. Example 3B shows modified ice-crystal shapes if a water-ice mixture is frozen into a film in combination with 0.01 wt% of AFP.

- WO 92/22581 discloses AFPs from plants which can be used for
25 controlling ice crystal shape in ice-cream. This document also describes a process for extracting a polypeptide composition from intercellular spaces of plants by infiltrating leaves with an extraction medium without rupturing the plant cells.

WO 94/03617 discloses the production of AFPs from yeast and their possible use in ice-cream. WO 96/11586 describes fish AFPs produced by microbes.

5 Up till now, however the use of AFPs has not been applied to commercially available consumer products. One reason for this are the high costs and complicated process for obtaining AFPs. Another problem is that sources of the AFPs are either difficult to obtain in sufficient quantities (e.g. fish
10 containing AFPs) or are not directly suitable for use in food products.

The present invention aims to provide novel antifreeze polypeptides which have the advantage that they can easily be
15 obtained from an abundant natural source and which provide good properties to products in which they are used.

It has been found that antifreeze polypeptides which possess good recrystallisation inhibition properties can be obtained
20 from carrots. In particular it has been found that antifreeze polypeptides obtained from carrots show markedly better properties as compared to polypeptides isolated from other root vegetables. In particular the antifreeze polypeptides of the invention are capable of providing good recrystallisation
25 inhibition properties without significantly changing the crystal shape of the ice-crystals, therewith possible leading to more favourable properties e.g. soft ice-cream.

Applicants have found that the effective antifreeze
30 polypeptides from carrots are generally characterised by an apparent Molecular Weight on SDS-PAGE of 36 kDa. Accordingly in a first aspect the invention relates to antifreeze

polypeptides which can be obtained from carrots and which have an apparent molecular weight on SDS-PAGE of 36 kDa.

In this context it will be clear to the skilled person that due to variation e.g. in SDS PAGE, the apparent molecular weight can only be determined with some variation in the results. For the purpose of the invention these variations e.g. from 30 to 40 kDa or from 34 to 38 kDa are also embraced within the scope of the term "apparent Molecular Weight of 36 kDa".

Applicants also have found that the effective anti-freeze polypeptides according to the invention comprise fragments having an amino acid sequence as represented in the examples.

Accordingly in a second aspect the invention relates to polypeptides comprising one or more fragments (A-E) having an amino acid sequence as follows:

- (A) LEU-PRO-ASN-LEU-PHE-GLY-LYS
- (B) ILE-PRO-GLU-GLU-ILE-SER-ALA-LEU-LYS
- (C) LEU-THR-X-LEU-ASP-LEU-SER-PHE-ASN-LYS
- (D) SER-LEU-ARG-LEU-SER-SER-THR-SER-LEU-SER-GLY-PRO-VAL-PRO-LEU-PHE-PHE-PRO-GLN-LEU-X-LYS
- (E) X-X-GLU-VAL-ILE-PRO-X-GLN-LEU-SER-THR-LEU-PRO-ASN-LEU-LYS

Preferably the AFPs of the invention comprise all of the partial sequences (A-E).

The complete amino acid sequence of the preferred AFP of the invention is represented below. Accordingly, in a third aspect the invention relates to an anti-freeze protein having an amino acid sequence as shown in Listing 1:

```

10      ATGAATATTGAATCATCTTCTGCGCTATTTTGTGCATATGCATGATTTTCCTCTGCGCTT
      13 ----- 72
      a  M N I E S S F C F I L C I C M I F L C L -
      CCAAACCTCTCTGCATCACAAGATGCAACAACGACAAGCAAGCTTTACTCCAAATC
15      73 ----- 132
      a  P N L S A S Q R C N N N D K Q A L L Q I -
      AAAACAGCCTTGAAAAACCCACCATTACAGACTCATGGGTGTGAGACGAGATTGTTGT
20      133 ----- 192
      a  K T A L K N P T I T D S W V S D D D C C -
      GGTGGGAOCTAGTGAATGTGACGAAACGACCAACGSCATAATTCCCTCATAATTCAA
25      193 ----- 252
      a  G W D L V E C D E T S N R I I S L I I Q -
      GACGACGAAGCTCTCACCGGCCAAATCCACCTCAGGTGGGAGACCTACCATACCTCCA
30      253 ----- 312
      a  D D E A L T G Q I P P Q V G D L P Y L Q -
      GCCTTATGGTTCGGTAAACTCCCAATCTTTTGGAAAAATCCGAGAAGAAATTTGCA
35      313 ----- 372
      a  A L W F R K L P N L F G K I P E E I S A -
      CTCAAAGACCTAAATCCCTCAGACTCAGCTCGACGAGTCTCAGTGGCCCTGTCCCTTA
40      373 ----- 432
      a  L K D L K S L R L S S T S L S G P V P L -
      TTCTTCCCTCAGCTTACGAACTAAGTTGTTAGACTTATCGTTTAAACAACTTTTGGGT
40      433 ----- 492
      a  F F P Q L T K L T C L D L S F N K L L G -

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          GTAATCGCTCCTCAGCTTTCCACTCTTCGGAACCTTAAAGCCCTGCACCTAGAACGTAAC
493 ----- 552
a  V I P P Q L S T L P N L K A L H L E R N -
5
          GAACACCGGTGAAATCCCGATATCTTTGGGAATTTTGGTGGATCCCCGGACATATAT
553 ----- 612
a  E L T G E I P D I E G N F A G S P D I Y -
10
          CTTTCGCATAACCGAGCTCACCGGTTTGTTCCTCCAAACTTTTGCTAGAGCAGATCCAATT
613 ----- 672
a  L S H N Q L T G F V P K T F A R A D P I -
          AGGCTCGACTTCTCAGGGAACAGACTAGAAGGTGATATTTCAATCTTGTTTGGGCCTAAA
673 ----- 732
a  R L D F S G N R L E G D I S F L F G P K -
15
          AAACGCTTGGAAATGCTAGATTTTCAGGAAACGTGCTTAGTTTCAATTTCTCCAGGGTG
733 ----- 792
a  K R L E M L D F S G N V L S F N F S R V -
20
          CAGGAGTTTCCACCCTCTTTGACATACTTAGACTTGAACCATACCGAGTCAGCGGAAT
793 ----- 852
a  Q E F P P S L T Y L D L N H N Q I S G S -
25
          CTGTGCGAGTGAATTGGCTAAATTGGACCTGCAGACATTTAACGTCAGTGATAATAATCTC
853 ----- 912
a  L S S E L A K L D L Q T F N V S D N N L -
30
          TGCAGCAAGATTCCACAGGGGGAAACCTCCAGAGATTGCAACGTCACGGCTATCTCCAC
913 ----- 972
a  C G K I P T G G N L Q R F D R T A Y L H -
          AACAGTTGCTTGTGTGGTGCTCCATTGCCAGAATGCTAG
973 ----- 1011
a  N S C L C G A P L P E C * -

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Listing 1

40

Also embraced within the invention are isoforms and derivatives of the above mentioned polypeptides which still possess the antifreeze properties. Preferable the derivatives show at least 75% homology with the polypeptide of Listing 1 45 or the polypeptide comprising the partial sequences (A-E),

more preferred more than 85%, most preferred more than 95%.
For the purpose of the invention the term derivative also
embraces modified polypeptides which still possess the
antifreeze properties, for example glycosylated forms of the
5 above polypeptides.

Also embraced within the invention are nucleotide sequences
encoding the amino acids as described above. In particular
the invention relates to nucleotide sequences of Listing 1
10 and alleles thereof.

Also embraced within the invention are nucleotide fragments
derived from the coding region that are capable of
hybridizing to related genes that code for anti-freeze
15 peptides.

Although the proteins of the invention can easily directly be
isolated from carrots, also genetic manipulation techniques
may be used to produce the proteins described in the
20 invention.

An appropriate host cell or organism would be transformed by
a gene construct that encodes the desired polypeptide. The
nucleotide sequence coding for the polypeptide can be
25 inserted into a suitable expression vector containing the
necessary elements for transcription and translation and in a
manner that they will be expressed under appropriate
conditions (eg in proper orientation and correct reading
frame and with appropriate targeting and expression
30 sequences). The methods required to construct these
expression vectors are well known to those skilled in the
art.

A number of expression systems may be utilised to express the polypeptide coding sequence. These include, but are not limited to, bacteria, yeast, insect cell systems, plant cell culture systems and plants all transformed with the appropriate expression vectors. Yeast, plants and plant culture systems are preferred in this context.

A wide variety of plants and plant cell systems can be transformed with the nucleic acid constructs of the polypeptides. Preferred embodiments would include, but are not limited to, maize, tomato, tobacco, carrots, strawberries, rape seed and sugar beet.

One preferred embodiment of the invention relates to the use of AFPs of the invention for increasing the frost tolerance of plants. This case for example be done by the above method whereby the plants are transformed to ensure (increased) production of the AFPs of the invention, therewith increasing the frost tolerance of said plants.

The invention also relates to antibodies which specifically bind an (epitope of the) polypeptides of the invention. Also embraced are polypeptides which are immunologically related to the polypeptides as determined by its cross reactivity with an antibody raised against the above polypeptides.

Based on the above information it is also possible to genetically modify other natural sources such that they produce the advantageous AFPs as identified here-above.

Preferably those AFPs are chosen which have significant ice-recrystallisation inhibition properties. A suitable test for determining the recrystallisation inhibition properties is indicated in example I. Also preferably AFPs in accordance to 5 the invention provide a ice particle size in the frozen product (mean crystal length) upon recrystallisation of less than 50 μm , more preferred from 5 to 40 μm .

The AFPs can conveniently be used in several products, 10 preferably in food products which are frozen or intended to be frozen. Carrots which comprise the AFP at naturally occurring levels are not embraced within the scope of the invention. However, food product containing (parts) of carrots are embraced within this term. Also embraced are 15 carrots which have been transformed to over express the AFP of the invention i.e. which contain the AFP at significantly higher levels than non-transformed carrots.

Examples of such food products are: frozen food products such 20 as vegetables, sauces, soups, snacks, frozen confectionery products such as ice-cream or water-ice, dairy products etc.

The preferred products wherein the AFPs are used are or frozen vegetables or frozen confectionery products such as 25 ice-cream or water-ice. Preferably the level of AFPs is from 0.00001 to 0.5 wt% based on the final product.

If dry-mixes or concentrates are used, the concentration may be higher in order to ensure that the level in the final 30 frozen product is within the above ranges. Surprisingly it has been found that compositions of the invention can contain very low amounts of AFPs while still being of good quality.

Preferred levels of AFP are from 0.00001 to 0.5 wt%, more preferred 0.00005 to 0.3 wt%, most preferred 0.0001 to 0.2 wt%.

5

For the purpose of the invention it is not necessary to add the AFP in purified form to the food product. Also possible is to add a composition comprising AFPs e.g. an extract of the natural material which produces the AFP.

- 10 Also it is possible to modify the food product such that the AFP is produced in situ e.g. by adding genetically modified micro-organisms which are capable of producing the AFP in the food product, or even to genetically modify the food product (e.g. the vegetable) such that (the vegetable) in itself it
- 15 is capable of producing the AFP in situ.

For the purpose of the invention the term frozen confectionery product includes milk containing frozen confections such as ice-cream, frozen yoghurt, sherbet,

20 sorbet, ice milk and frozen custard, water-ices, granites and frozen fruit purees.

Preferably a the level of solids in the frozen confection (e.g. sugar, fat, flavouring etc) is more than 3 wt%, more

25 preferred from 10 to 70wt, for example 40 to 70 wt%.

Frozen confectionery products according to the invention can be produced by any method suitable for the production of frozen confectionery. Especially preferably however all the

30 ingredients of the formulation are fully mixed before the freezing process starts.

EXAMPLES

Example I

- 5 Carrots (*Daucus carota* cv Autumn King) were grown in individual pots. When plants were approximately twelve weeks old, they were transferred to a cold room and held at 4°C in constant light during 4 weeks for cold-acclimation. Plants were watered three times a week.
- 10 Fresh tissue of the carrots were ground with a pestle and mortar (cooled to 4°C) in an equal volume buffer A (10mM EDTA, 20 mM Ascorbic acid, buffered with Tris to pH 7.4) held on ice. The homogenates were filtered through one layer of 15 muslin and kept on ice prior to further use.
- As a comparison several other root-plants were grown and homogenates prepared from the roots as above.
- 20 Anti-freeze activity was measured using a modified "splat assay" (Knight et al, 1988). 2.5 µl of the solution under investigation in 30% (w/w) sucrose was transferred onto a clean, appropriately labelled, 16 mm circular coverslip. A second coverslip was placed on top of the drop of solution 25 and the sandwich pressed together between finger and thumb. The sandwich was dropped into a bath of hexane held at -80°C in a box of dry ice. When all sandwiches had been prepared, sandwiches were transferred from the -80°C hexane bath to the viewing chamber containing hexane held at -6°C using forceps 30 pre-cooled in the dry ice. Upon transfer to -6°C, sandwiches could be seen to change from a transparent to an opaque

appearance. Images were recorded by video camera and grabbed into an image analysis system (LUCIA, Nikon) using a 20x objective. Images of each splat were recorded at time = 0 and again after 30-60 minutes. The size of the ice-crystals in both assays was compared. If the size at 30-60 minutes is similar or only moderately increased (say less than 20% increased, more preferred less than 10% increased, most preferred less than 5 % increased) compared to the size at t=0, this is an indication of good ice-crystal recrystallisation inhibition properties.

Results: from the sandwich splat assay test it appeared that samples from carrot roots, carrot stem and carrot leaves possess significant ice-recrystallisation inhibition properties, whereby the roots and leaves are most active. As a comparison a sample of non-acclimated carrot roots was tested, which showed significant less activity. For the following examples root tissue was used for further testing on carrots.

20

As a comparison several other vegetable roots were investigated by means of the sandwich splat assay test in 30% sucrose. Among these vegetables were turnip, kale, brussels sprout, wintergreen cabbage, rape, pak choi, parsnip and strawberry. None of these sources of material provided significant ice-recrystallisation inhibition activity.

Example II

Carrot root tissue was homogenized in three volumes (w/v) buffer (20mM ascorbic acid, 10 mM EDTA, 50 mM Tris/HCL, pH 7.2) in a pre-cooled pestle and mortar and filtered through one layer of muslin. The filtrate was centrifuged at 6,000 g, ten minutes at 4°C; the supernatant was collected and centrifuged at 100,000g for 1 hour at 4°C. The 100,000 g supernatant from this step is termed the soluble fraction and the pellet the microsomal fraction.

The supernatant was applied to a 30 ml fast flow Q Sepharose (Pharmacia) column pre-equilibrated in 50 mM Tris/HCL pH 7.4 at a flow rate of 5 ml/min supplied by a HiLoad pump P-50 controlled by a Gradifrac low pressure chromatography system (Pharmacia) at 4°C and the eluate monitored at OD 280 by a UV monitor (Monitor UV1, Pharmacia) recorded on a chart recorder (REC 102, Pharmacia). 5 ml fractions were collected. The column was washed with 50mM Tris/HCL pH 7.4 at the same flow rate until the OD 280 returned to zero. A 150ml gradient of 0-0.4 M NaCl in Tris/HCL pH 7.4 was then applied followed by a 2 M NaCl column wash. Eluate fractions were subjected to the splat assay as in example I.

Fractions containing anti-freeze activity as evidenced by recrystallisation inhibition were pooled and concentrated using polyethylene glycol as follows: the fractions were transferred in 10kDa cut off dialysis tubing (Sigma) which had been washed in tap water, boiled in 50mM EDTA pH 7.5 for 10 minutes and rinsed in milli Q water. The dialysis tubing containing the sample to be concentrated was covered with solid polyethylene glycol compound Mol. Wt. 15,000 - 20,000

(Sigma) and incubated at 4°C for up to 4 hours or until the sample volume inside the dialysis tubing had reduced up to 10 fold.

5 The pooled concentrate from the Q sepharose column was applied either to a phenyl Sepharose column, a SMART superdex 75 gel permeation column or an FPLC superdex 75 gel permeation column.

10 Carrot root anti-freeze proteins were purified by gel permeation chromatography as follows:

20µl aliquots of sample were applied to a SMART superdex 75 column (Pharmacia) pre-equilibrated in 50mM Tris/HCl pH7.4 containing 0.15M NaCl (Buffer E) at a flow rate of 40µl/min and components separated by gel permeation at the same flow rate in equilibration buffer. The eluate was monitored at OD 280 and OD 215. 80µl fractions were collected between 0.85 and 0.89ml, 40µl fractions between 0.89 and 1.24ml and 100µl fractions between 1.24 and 3.0 ml. The void volume (Vo) of the column was 0.91 ml as determined by the retention volume of a solution of Blue Dextran. The superdex column was calibrated by application of 10µl of a solution containing 5mg/ml BSA (Mr 66kDa, retention (Ve)=1.02 ml), 3mg/ml 25 Carbonic anhydrase (Mr 29 kDa, Ve=1.22 ml), 2mg/ml Cytochrome C (Mr 12.4 kDa, Ve=1.41 ml) and 2mg/ml Aprotinin (Mr 6.5 kDa, Ve=1.59 ml) and a standard curve plotted of Ve/Vo against log Mr. Fractions containing anti-freeze activity were identified by the splat assays as described in Example I, with an 30 activity peak that showed a retention volume of 1.16 ml and an apparent molecular weight of 40 kDa. These measurement

confirmed that the 36 kDa band from cold acclimatized carrots was an anti-freeze peptide.

SDS-PAGE was carried out according to Laemmli (1970) using the Biorad mini system. Samples to be analyzed by SDS-PAGE were dissolved in SDS-PAGE sample buffer (Laemmli 1970), heated for 5 minutes at 100°C on a dry heating block (Techne) and centrifuged for 3 minutes at 10,000g at room temperature. Samples (10-50µl) were applied to mini-gels (Biorad, 0.75, 1.0 or 1.5mm thickness, 10,12,15% acrylamide or 10-20% gradient acrylamide (pre-poured from Biorad)) and electrophoretically separated. Separated polypeptides were fixed and stained in the gel either with Coomassie blue (0.1% {w/v} Coomassie Brilliant Blue in acetic acid/methanol/miliQ water {5:4:31, 15 by vol}) or silver stained using the Biorad silver stain kit according to the manufacturer's instructions. Gels were dried between two sheets of Gelair cellophane in a Biorad gelair dryer according to the manufacturer's instructions. Sigma high and low range molecular weight marker kits were used according to the manufacturer's instructions for determination of apparent M_r on SDS-PAGE.

The ion exchange chromatography was carried out with cold acclimatized carrot root and non-cold acclimatized carrot root. The resulting gel SDS-PAGE gels showed the presence of a 36kDa band in the cold acclimatized sample. This band was much less abundant in the non-cold acclimatized root. This 36kDa band was hence attributed to anti-freeze activity.

Example III

For protein sequencing, the 36kDa carrot root protein was purified as described in the previous example and then to ensure further purification the sample to be sequenced was excised from the SDS PAGE gel and then proteolytically digested *in situ* in the polyacrylamide gel slice.

Preparations of largely pure 36 kDa protein, that still had some minor contaminating proteins, were loaded onto a 12% polyacrylamide gel. Three lanes each with 2 μ g of protein were loaded and electrophoresed in the gel until the dye front reached the bottom of the gel. The gel was then stained in 0.2% uocmassie brilliant blue (w/v), 30% methanol (v/v), 1% acetic acid (v/v) for 20 minutes and then destain with 30% methanol until the protein bands could be visualised. The 36 kDa band was identified by comparison with molecular weight markers loaded into adjacent lanes and the band from each lane was excised with a scalpel blade, taking care to exclude contaminating bands.

The gel slices were transferred to a clean eppendorf tube and washed twice with 0.5ml of 50% acetonitrile (v/v), 100mM Tris/Cl, pH 8.5. The washing removed some of the uocmassie stain and also partially dehydrated the gel slices. The gel slices were then removed from the tube and subjected to air drying on the laboratory bench until they had shrunk significantly and started to curl up. They were then transferred back to the eppendorf and rehydrated with firstly, 10 μ l of 100mM Tris/Cl, pH 8.5 containing 1 μ g of endoproteinase Lys C (Boehringer Mannheim). This is a proteinase that specifically cleaves polypeptide chains on

the carboxy terminal side of lysine residues. Further Tris buffer was added to the gel slices until they were fully rehydrated and they were then incubated at 37°C for 16 hours.

- 5 After incubation 1μl of trifluoroacetic acid was added to the tube to stop the reaction and then the gel slices were washed twice with 0.3ml of 60% acetonitrile (v/v), 0.1% TFA (v/v) at 30°C for 30 minutes. This was to again partially dehydrate the gel slices causing them to shrink and elute the peptides
- 10 that had been generated. The supernatant was transferred to another clean eppendorf tube and then dried in a centrifugal evaporator for 2 hours until the sample was near dryness and resuspended to a volume of 0.1ml with 0.1% TFA.
- 15 The peptides were then separated by reversed phase HPLC on a Smart micropurification system (Pharmacia). The peptide digest was loaded onto a C18 column (2.1 x 100 mm) equilibrated in 0.1% TFA (Solvent A) at a flow rate of 0.1ml min. The column was then eluted with a gradient of 0 - 70% of
- 20 Solvent B (90% acetonitrile v/v, 0.085% TFA v/v) over 70 minutes at the same flow rate. The optical density was monitored at 214 nm and individual peptide peaks were collected in the fraction collector by manual stepping. Polypeptides were sequenced by loading onto a model 492
- 25 Perkin Elmer protein sequencer using the liquid phase chemistry cycles as recommended by the manufacturer.

Several polypeptide fragments (A-E) were analyzed in the 36 kDa band and had sequences substantially homologous to:

5

(A) LEU-PRO-ASN-LEU-PHE-GLY-LYS

(B) ILE-PRO-GLU-GLU-ILE-SER-ALA-LEU-LYS

10 (C) LEU-THR-X-LEU-ASP-LEU-SER-PHE-ASN-LYS

(D) SER-LEU-ARG-LEU-SER-SER-THR-SER-LEU-SER-GLY-PRO-VAL-
PRO-LEU-PHE-PHE-PRO-GLN-LEU-X-LYS15 (E) X-X-GLY-VAL-ILE-PRO-X-GLN-LEU-SER-THR-LEU-PRO-ASN-LEU-
LYS

Example IV-a carrot cell culture

A carrot cell suspension culture line (NOR) was obtained from the Department of Biochemistry and Molecular Biology, University of Leeds. The culture was maintained by subculturing 10 ml of the culture into 90 ml of fresh Murashige and Skoog medium (Sigma) containing 25 g/l sucrose and 1 mg/l 2,4-D every seven days. Cultures were incubated in an orbital shaking incubator at 150 rpm at 25°C in the dark.

10

The NOR culture was cold treated as follows:

NOR cultures were transferred to 4°C after 4d and 7d of growth at 25°C. Cultures were harvested at t=0, t=7d and t=14d. In addition to harvesting, the packed cell volume (PCV) was determined for each culture at each time point.

The media samples from NOR suspension cultures were analyzed as follows. Approximately 1/10th of the volume of a frozen aliquot of conditioned suspension culture medium was allowed to defrost. The defrosted (freeze concentrated) portion was removed and tested for activity by sandwich splat assays as described in Example I. Medium from cold acclimated cultures was found to contain significantly more activity than medium from non-cold acclimated cultures.

The cold acclimated NOR carrot medium was buffered by addition of 100µl of 1M Tris/HCl pH 7.4. Purification of activity was then performed by ion exchange and gel permeation chromatography using a method based on that in Example II: the buffered medium was applied to a 1 ml Q Sepharose column (Pharmacia) at a flow rate of 1 ml/min and

bound molecules eluted with 3 ml aliquots of 500 mM Tris/HCl pH 7.4 containing concentrations of NaCl starting at 0.1 M and increasing to 0.5 M in 0.1 M steps. 1 ml fractions were collected and tested for activity as in Example I.

5

The antifreeze activity in the active ion exchange fractions was purified by gel permeation chromatography as follows. The active fraction from above was acetone precipitated and the pellet resuspended in 50 μ l 50mM Tris/HCl + 0.15 M NaCl, pH 7.2. This was then centrifuged at 10.000 g for 10 minutes, and 20 μ l loaded onto a Superdex 75 gel permeation column on the Pharmacia SMART system. The flow rate was 40 μ l/min and the mobile phase was 50mM Tris/HCl + 0.15M NaCl, pH 7.2. 80 μ l fractions were collected and splatted. Activity was detected in fractions corresponding to a retention of 1.16 ml.

Further isolation of the active proteins can be done by SDS PAGE analysis in line with Example II.

Example IV-b carrot root culture

Carrot root cultures were initiated as follows.

- 5 For each individual culture 10 surface sterilised *Daucus*
carote cv Autumn King seeds were placed into 100 ml MS medium
containing 25 g/L sucrose and 0.5 g/L MES in sterile 250 ml
Erlenmyer flasks. Seeds were germinated by shaking at 150 rpm
in the dark at 25°C for 3 weeks. Leaves and shoots were then
10 aseptically removed. The roots were replaced into 100 ml
fresh medium and incubated with shaking for a further 2
weeks.

- Homogenates were prepared from cold treated and non-cold
15 treated root cultures as follows. Fast frozen roots were
ground up 3x in liquid nitrogen in a cold mortar and pestle
then transferred to a further chilled mortar and pestle and
ground up with 0.5x volume of ice-cold 50mM Tris.HCl + 10 mM
EDTA pH 7.4 containing 30 % w/w sucrose. Homogenates were
20 centrifuged at 10.000 g for 10 minutes at 4°C and the
supernatant tested for activity as in Example I.
Significantly more activity was detected in cold treated root
cultures than in non-cold treated root cultures.

Example V preparation of ice-cream

Root extract from cold acclimatised carrot roots was prepared by scrubbing freshly pulled cold acclimatised (as in example 5 I) carrots in cold water. The tops are removed and the juice extracted employing a domestic juice extractor (Russell Hobbs, model no 9915). The juice was frozen in 1 litre blocks and stored at -20°C prior to collection for use in ice cream trials. The carrot AFP juice was added to the following ice cream formulation:

INGREDIENT	parts by weight
Skimmed Milk Powder	10.000
Sucrose	13.000
MD40	4.000
Locust Bean Gum	0.144
Genulacta L100	0.016
MGP	0.300
Butteroil	8.000
Vanillin	0.012
Water	64.528
Carrot Extract (from cold acclimated carrots containing 1-10 mg AFP per kg)	4.472

Ice-cream was prepared by freezing the above formulation and aeration to 106% overrun.

Measurements were made on fresh sample and on samples which had been abused by storage at -10°C for a period of 10 days. As a comparison a sample without carrot extract was measured in the same way. The measurements were done as follows:

Samples were equilibrated at -18°C in a Prolan Environmental cabinet for approximately 12 hours. Three samples were chosen representatively from each batch of ice cream and a slide was prepared from each in a Cryostat temperature control cabinet by smearing a thin layer of ice cream from the centre of each block onto a microscopic slide. A single drop of white spirit was applied to the slide and a cover slip was then applied. Each slide, in turn, was then transferred to a temperature controlled microscope stage (Leit LaborLux S, Leica x10 objective, temperature -18°C). Images of ice-crystals (about 400 individual ice-crystals) were collected and relayed through a video camera (Sanyo CCD) to an image storage and analysis system (LEICA Q520MC).

The stored ice crystal images were highlighted manually by drawing around the perimeter which then highlights the whole crystal. Images of the highlighted crystals were then measured using the image analysis software which counts the number of pixels required to complete the longest straight line (length), shortest straight line (breadth), the aspect ratio (length/breadth). The data for each individual ice crystal of a batch of ice cream was imported into a spreadsheet where analysis of the data set was carried out to find the mean, and standard deviation.

The ice Cream Hardness Measurements were carried out using a Hounsfield H10KM Universal Tester, a Hounsfield 100N Load Cell and a 10cm Cylindrical Stainless steel probe. The ice-cream samples were prepared by 16 Hour incubation of 486ml 5 ice cream blocks in a Prolan Temperature Control Cabinet set at -18 °C.

The ice cream block was removed from Prolan temperature control cabinet and placed the Hounsfield H10KM Universal 10 Tester. The 10cm cylindrical probe was pushed into the ice cream block at a constant rate of 400mm/min to a depth of 20mm. The maximum force recorded during the compression was used and expressed as the ice cream Hardness. If cracking or brittle fracture of the sample was observed this was 15 indicated in the right hand column

The following results were obtained

Sample	Ice Crystal Size Parameters				Material Properties	
	Mean Crystal Length / μm	Mean Crystal Breadth / μm	Mean Crystal Shape Factor / -	Mean Crystal Aspect Ratio / -	Hardness / N	Brittle Fracture observation
Carrot AFP - fresh	26.79 \pm 1.3	19.00 \pm 0.9	1.15 \pm 0.013	1.43 \pm 0.024	40.8	Yes
Carrot AFP - Abused	33.48 \pm 1.3	24.61 \pm 0.9	1.13 \pm 0.013	1.37 \pm 0.020	59.9	Yes
Cont.- Fresh	33.67 \pm 1.1	24.79 \pm 0.8	1.12 \pm 0.008	1.38 \pm 0.018	27.3	No
Cont.- Abused	61.77 \pm 2.7	46.54 \pm 2.0	1.11 \pm 0.010	1.37 \pm 0.020	32.7	No

5 This proves that carrot AFP has good ice recrystallisation inhibition properties.

Example VI

The peptide sequences shown in Example III were analyzed as to their suitability for degenerate oligonucleotide primer design. Part of Peptide D (GLY-PRO-VAL-PRO-LEU-PHE-PHE-PRO) was chosen and the primer cp3 (GGI CCI GTI CCI YTI TTY TTY CC, where I= inosine and Y=C or T) was synthesized (Genosys).

- 10 First strand cDNA was prepared from 5 µg cold acclimated (1 month as in example I) carrot root RNA using Superscript Reverse Transcriptase (Stratagene) and an oligonucleotide primer OG1(GAGAGAGGATCCTCGAG(T)¹⁵) according to the manufacturer's instructions. 1% of the first strand cDNA reaction was used as template, together with cp3 and OG1 primers, in subsequent PCR. The reactions were carried out in a thermal cycler using Taq DNA polymerase (Gibco BRL) for 30 cycles (1 minute at 94 °C, 1 minute at 50 °C and 1 minute at 72 °C) according to the manufacturer's
- 20 instructions. All primers were used at a concentration of 1 µM. The resulting ~800 bp PCR product was gel purified and cloned into the pTAq vector (R&D Systems) according to the manufacturer's instructions. The cloned cp3 PCR product was sequenced using the dideoxy sequencing method employed by
- 25 the Sequenase kit (USB). The cp3 nucleotide sequence and deduced amino acid sequence were substantially similar to:

5 a 1 GGCGCGGTGCGCGTGTTCTTCCCTCAGCTTAGGAACTAACTTGTTTAGACTTATCGTTT 60
G P V P L L F F P Q L T K L T C L D L S F -

10 a 61 AACAAACTTTTGGGTGAATCCCTCCTCAGCTTTCAGCTCTTCGGAAGCTTAAAGCCCTG 120
N K L L G V I P P Q L S T L P N L K A L -
121 CACTTAGAACGTAAACGAACCTACCGGTGAAATCCCGATATCTTTGGGAATTTTGTCTGGA 180
a H L E R N E L T G E I P D I F G N F A G -

15 181 TCCCCGGACATATATCTTTGCATAACCAAGCTCACCGGTTTGTTCCTCCAAACTTTTGCT 240
a S P D I Y L S H N Q L T G F V P K T F A -

20 241 AGAGCAGATCCAATTAGGCTCGACTTCTCAGGGAACAGACTAGAAGGTGATATTTCACTTC 300
a R A D P I R L D F S G N R L E G D I S F -

25 301 TTGTTTGGGCCTAAAAACGCTTGGAATGCTAGATTTTTCAGGAAACGTGCTTAGTTTC 360
a L F G P K K R L E M L D F S G N V L S F -

30 361 AATTTCTCCAGGGTGAGGAGTTTCCACCTCTTTGACATACTTAGACTTGAACCATTAAC 420
a N F S R V Q E F P P S L T Y L D L N H N -

35 421 CAGATCAGCGGAAGTCTGTGAGTGAATTGGCTAAATTGAGACTGCAGACATTTAACGTC 480
a Q I S G S L S S E L A K L D L Q T F N V -

40 481 AGTGATAATAATCTCTGCGGCAAGATTCCAACAGGGGAAACCTCCAGAGATTGACCGGT 540
a S D N N L C G K I P T G G N L Q R F D R -

45 541 ACGGCGCTATCTCCACAACAGTTGCTTGTGTGTGCTCATTGCCAAGATGCTAGTTACCA 600
a T A Y L H N S C L C G A P L P E C *
501 TGCAAAATGTGCTTAAAGTTATCTTTGTAATGAGATATATTATGCAGCTCAAGGCAGAG 660

```

      CAATAAGTTTTCTAATTTGTTATAGTAAGATATTATTGTATTTACAGAAAGTGTCTAC
661 ----- 720
5    TAGGATTCGTAATATATTATAATTGCTCATAATTGTATCTGTTTAACTCTGTAATCGAAAA
721 ----- 780
      ACCTTTATGTATTGGTTTGACACTTTTGAGCTTTAAAAAAAAAAAAAAAA
781 ----- 829
10

```

Listing II

In order to obtain the full coding region for the carrot
 15 AFP, a cDNA library was constructed. A poly (A)+ quick
 column (Stratagene) was used to purify mRNA from 500 µg CA
 (1 month) carrot total RNA, according to manufacturer's
 instructions. All resulting poly (A)+ RNA was used for cDNA
 synthesis and subsequent library construction using the
 20 lambda ZAP vector kit (Stratagene). 1×10^5 recombinant
 phage clones were screened by hybridization using the *cp3*
 PCR product as a ^{32}P labelled probe.

Positive plaques were screened to purity and phage-mids
 25 excised before the inserts were characterised by DNA
 sequence analysis. Two cDNA clones were sequenced to
 completion. Although the 5' and 3' untranslated regions
 contained some sequence variability, the coding regions
 were identical. The coding regions of the two cDNA clones
 30 were substantially similar to:

13 ATGAATATTGAATCATCTTTCTGCCCTATTTTGTGCATATGCATGATTTTGCTCTGCCTT 72
 M N I E S S F C P I L C I C M I F L C L -
 5 a
 73 CCAAACCTCTCTGCATCACAAGATGCAACAACGACGAAGCTTTACTCCAAATC 132
 P N L S A S Q R C N N N D K Q A L L Q I -
 10 a
 133 AAAACAGCCTTGAAAAACCCACCATTACAGACTCATGGGTGCAGACGACGATTTGTG 192
 K T A L K N P T I T D S W V S D D D C C -
 15 a
 193 GGTGGGACCTAGTGAATGTGACGAAACGAGCAACGGCATAATTCCCTCATAATTCAA 252
 G W D L V E C D E T S N R I I S L I I Q -
 20 a
 253 GAGGACGAAGCTCTCACCGGCCAAATCCACCTCAGGTGGGAGACCTACCATACCTCCAA 312
 D D E A L T G Q I P P Q V G D L P Y L Q -
 25 a
 313 GCCTTATGGTTCGGTAAACTCCCAATCTTTTCGGAAAAATCCAGAAGAAATTTCTGCA 372
 A L W F R K L P N L E G K I P E E I S A -
 30 a
 373 CTCAAAGACCTAAAATCCCTCAGACTCAGCTCGACCACTCTCAGTGGCCCTGTCCCTTTA 432
 L K D L K S L R L S S T S L S G P V P L -
 35 a
 433 TTCTTCCCTCAGCTTACGAAACTAAGTTGTTAGACTTATCGTTTAAACAACTTTTGGGT 492
 F F P Q L T K L T C L D L S F N K L L G -
 40 a
 493 GTAATCCCTCCTCAGCTTTCACCTCTTCGAACTTAAAGCCCTGCACTTAGAACGTAAAC 552
 V I P P Q L S T L P N L K A L H L E R N -
 45 a
 553 GAACCTCACCGGTGAAATCCCGATATCTTTGGGAATTTTGTGGATCCCGGCACATATAT 612
 E L T G E I P D I F G N F A G S F D I Y -
 50 a
 613 CTTTCGCATAACCAAGCTCACCGGGTTTGTTCGCAAACTTTGCTAGAGCAGATCCAATT 672
 L S H N Q L T G F V P K T F A R A D P I -
 55 a
 673 AGGCTCGACTTCTCAGGGAACAGACTAGAGGTGATATTCATTCTGTGTGGGCTAAAC 732
 R L D F S G N R L E G D I S F L F G P K -
 60 a
 733 AAACGCTTGGAAATGCTAGATTTTTCAGGAAACGTCTAGTTTCAATTTCTCCAGGGTG 792
 K R L E M L D E F S G N V L S F N F S R V -
 65 a
 793 CAGGAGTTTCCACCCCTCTTGACATACTAGACTGAACCATACAGATCAGCGGAAGT 852
 Q E F P P S L T Y L D L N H N Q I S G S -
 70 a
 853 CTGTGAGTGAATGGCTAAATGGACCTGCAGACATTAAAGTCAGTGATAATAATCTC 912
 L S S E L A K L D L Q T F N V S D N N L -
 913 TGCGGCAAGATTCCAACAGGGGGAAACCTCCAGAGATTGCAACGTAACGGCTATCTCCAC 972
 C G K I P T G G N L Q R F D R T A Y L H -
 AACAGTTGCTTGTGGTGTCTCCATTGCCAGAAATGCTAG 1011
 N S C L C G A P L P E C * -

Listing I

Partial sequence analysis of 4 other clones also indicated that they contained the same coding region as the fully sequenced clones and thus all the positives from the 5 library screen were likely to represent transcripts from the same gene. The existence of only one copy of the AFP gene in the carrot genome was further substantiated by the fact that Southern analysis of restriction enzyme digested carrot genomic DNA suggested that only one fragment 10 hybridized to the probe.

Example VII

In order to prove that the carrot cDNA as shown in example
5 VI represented an AFP, expression of the coding region was
carried out as follows. One of the cDNAs was first cloned
into an intermediate pUC plasmid vector (Messing, 1983)
containing a double CaMV 35S promoter (Guerineau, J. F.,
Woolsten, S., Brooks, L., and Mollineaux, P. (1988))
10 expression cassette, and then into a binary vector, as
described below. All enzymes used were supplied by Gibco
BRL and used according to the manufacturer's instructions.

The pBluescript phagemid (Stratagene) containing the cDNA
15 clone was digested with *Xho* I and the recessed 3' termini
filled in using the Klenow fragment of DNA polymerase I.
The cDNA fragment was then released from the vector by
digestion with *Eco* RI. The *Eco* RI/blunt cDNA fragment was
then cloned into the *Eco* RI/blunt digested intermediate pUC
20 plasmid vector. The CaMV 35S-cDNA expression cassette was
then subcloned as a partial *Hind* III fragment into
Hind III-cut pBin 19 binary vector (Bevan 1984). The binary
vector construct was then introduced into tobacco using
Agrobacterium mediated transformation (as described in
25 Draper, J., Scott, R., Armatage, P., and Walden, R.
(1988)).

Transgenic tobacco callus was analyzed for expression of
recrystallisation inhibition activity as soon as sufficient
30 kanamycin resistant material was regenerated. Small scale
protein extracts were made from several independent

kanamycin resistant calli plus some wild type tobacco callus. Approximately 2 g tissue was ground up in 1-2 mls sucrose buffer (30 % sucrose 50 mM Tris, 10 mM EDTA, 20 mM ascorbate, pH 7.2) using a mortar and pestle.

5 The solution was centrifuged at 10,000 xg for 2 minutes and the supernatant removed to a fresh tube. An aliquot of 3 µl of protein extract was tested for recrystallisation activity using the sucrose sandwich splat assay method of example I. All kanamycin resistant callus extracts tested

10 demonstrated recrystallisation inhibition activity.

Stable transgenic tobacco plants expressing the carrot AFP have also been produced. Leaf extracts from wild-type and transgenic tobacco plants have been subjected to northern

15 analysis using the AFP cDNA as a probe. The AFP message was only detectable in the transgenic tobacco plants. This suggests that the AFP message is stable in the greenhouse grown transgenic tobacco plants. When compared with the native carrot transcript, the tobacco AFP transcript

20 appears to be slightly bigger. This discrepancy can be explained by the method of construction of the AFP expression cassette. Because the *CaMV* 35S polyadenylation signal is most 3' in the construct, it is likely that this signal is used in the transgenic AFP message, giving rise

25 to a longer transcript. Leaf extracts from wild-type and transgenic tobacco plants have also been analyzed by western blotting using a carrot AFP antibody. A cross-reacting protein was only detected in the transgenic tobacco plants. Despite the difference in transcript size,

30 the protein produced in tobacco appears to be the same size as the native carrot AFP.

The above data provides the proof that the protein purified from carrot and the corresponding cDNA represent an active AFP.

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ANNEX

33
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CLAIMS

1. Polypeptides having antifreeze activity which can be obtained from carrots and which have an apparent molecular weight on SDS-PAGE of 36 kDa and isoforms or derivatives thereof which still possess antifreeze activity.
2. Polypeptides having antifreeze activity comprising one or more fragments (A-E) of the amino acid sequence as follows:
 - (A) LEU-PRO-ASN-LEU-PHE-GLY-LYS
 - (B) ILE-PRO-GLU-GLU-ILE-SER-ALA-LEU-LYS
 - (C) LEU-THR-X-LEU-ASP-LEU-SER-PHE-ASN-LYS
 - (D) SER-LEU-ARG-LEU-SER-SER-THR-SER-LEU-SER-GLY-PRO-VAL-PRO-LEU-PHE-PHE-PRO-GLN-LEU-X-LYS
 - (E) X-X-GLY-VAL-ILE-PRO-X-GLN-LEU-SER-THR-LEU-PRO-ASN-LEU-LYS

and isoforms or derivatives thereof which still possess antifreeze activity.

AMENDED SHEET

3. Polypeptides having antifreeze activity comprising the fragments (A-E) of claim 2.
4. Polypeptides having antifreeze activity having an amino acid sequence as represented in Listing 1 and isoforms and derivatives thereof which still possess antifreeze activity.
5. An isolated nucleic acid sequence encoding the antifreeze polypeptide of one or more of claims 1-4 and alleles thereof encoding polypeptides which still possess antifreeze activity.
6. An isolated nucleic acid sequence corresponding to the gene sequence of Listing I and alleles thereof encoding polypeptides which still possess antifreeze activity .
7. Method of obtaining polypeptides according to one or more of claims 1-4 whereby the polypeptide is isolated from cold-acclimatized carrots.
8. Method of obtaining polypeptides according to one or more of claims 1-4, whereby the polypeptide is expressed by a genetically modified organism.
9. Method according to claim 8, whereby the organism is a micro-organism, a plant or a cell culture.

AMENDED SHEET

10. An antibody capable of specifically binding the polypeptide of claim 1, 2, 3 or 4.
11. A polypeptide which has antifreeze activity that is immunologically related to the polypeptide of claim 1, 2, 3 or 4 as determined by its cross reactivity with an antibody of claim 10.
12. Food product comprising a polypeptide of claim 1, 2, 3, 4 or 11 with the proviso that the food product is not a carrot.
13. Food product of claim 12 being a frozen confectionery product or a frozen vegetable.
14. Method of producing a food product comprising an antifreeze polypeptide according to one or more claims 1, 2, 3 or 4, comprising the steps of
 - (a) adding to the food product a composition comprising said antifreeze polypeptide; or
 - (b) in situ production of said antifreeze polypeptide.
15. Use of the polypeptide of claims 1, 2, 3 or 4 for increasing the frost tolerance of plants.

2000-01-01 09:00

16. Micro-organisms, cell line or plant capable of expressing the polypeptide of claims 1, 2, 3 or 4, with the proviso that the plant is not an unmodified carrot plant.

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY

(Includes Reference to PCT International Applications)

ATTORNEY'S DOCKET NUMBER
F7371 (C)

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

CARROT ANTIFREEZE POLYPEPTIDES

the specification of which (check only one item below):

☐ is attached hereto.☐ was filed as United States application Serial No. _____ on _____
and was amended on _____ (if applicable)☒ was filed as PCT International application PCT/EP97/06181 on 6th November 1997
and was amended under PCT Article 19 on _____ (if applicable)

I hereby state that I have received and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. 119:

COUNTRY (if PCT indicate PCT)	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 USC 119
EUROPE	96308362.1	19th November 1996	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO

I hereby claim the benefit under Title 35, United States Code §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code §112. I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations §1.56 (a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application.

PRIOR U.S. PROVISIONAL APPLICATION(S) FOR BENEFIT UNDER 35 U.S.C. 119 (e):

APPLICATION NUMBER	DATE OF FILING (day, month, year)

I hereby claim the benefit under Title 35, United States Code §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code §112. I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations §1.56 (a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application.

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. 120.

U.S. APPLICATION(S)	STATUS (CHECK ONE)			
U.S. APPLICATION NUMBER	U.S. Filing Date	PATENTED	PENDING	ABANDONED
PCT APPLICATIONS DESIGNATING THE U.S.				
PCT APPLICATION NUMBER	PCT Filing Date	U.S. Serial Numbers Assigned (if any)		
PCT/EP97/06181	6th November 1997			

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY (continued)
(includes Reference to PCT International Applications)

ATTORNEY DOCKET NUMBER
F7371 (C)

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.
(List name and registration number)

James J. Farrell, Esq., Reg. 28,162
Ronald A. Koatz, Esq., Reg. 31,774
Neil Y. Gilbert, Reg. 35,156

Milton L. Honig, Esq., Reg. 28,617 Gerard J. McGowan, Jr., Esq., Reg. 29,412
Rimma Mitelman, Reg. 34,386 Matthew Boxer, Reg. 28,495
Edward A. Squillante, Jr. Reg. 38,319

Send correspondence to:
Patent Department
Unilever United States, Inc.
45 River Road, Edgewater, NJ 07020

Direct Telephone Calls to: (name and telephone #)
James J Farrell
(201) 943 7100 - ext. 2332

01

Full Name of Inventor	Family Name	First Given Name	Second Given Name
1 - CC	BYASS	Louise	Jane
Residence & Citizenship	City Alberta CAX	State or Foreign Country Canada	Country of Citizenship United Kingdom
Post Office Address	Post Office Address 30510707, 81 Avenue, Edmonton	City Alberta	State & Zip Code/Country T6E1Y2, Canada

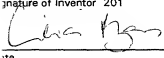
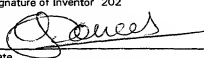
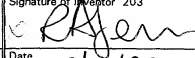
02

Full Name of Inventor	Family Name	First Given Name	Second Given Name
2 - CC	DOUCET	Charlotte	Juliette
Residence & Citizenship	City York GBX	State or Foreign Country United Kingdom	Country of Citizenship United Kingdom
Post Office Address	Post Office Address C/o The University of York, Department of Biology, The Plant Laboratory, Heslington	City York	State & Zip Code/Country YO1 5YW, United Kingdom

03

Full Name of Inventor	Family Name	First Given Name	Second Given Name
3 - CC	FENN	Richard	Anthony
Residence & Citizenship	City Bedford GBX	State or Foreign Country United Kingdom	Country of Citizenship United Kingdom
Post Office Address	Post Office Address 13 Ryton Close, Brickhill,	City Bedford	State & Zip Code/Country MK41 7XA, United Kingdom

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signature of Inventor 201	Signature of Inventor 202	Signature of Inventor 203
		
Date 14.10.99	Date 1.11.99	Date 9/11/99

204

Full Name of Inventor <u>A - CC</u>	Family Name <u>McArthur</u>	First Given Name <u>Andrew</u>	Second Given Name <u>John</u>
Residence & Citizenship	City <u>Bedford</u> <u>GBX</u>	State or Foreign Country United Kingdom	Country of Citizenship United Kingdom
Post Office Address	Post Office Address C/o Unilever Research Colworth, Colworth House, Sharnbrook	City Bedford	State & Zip Code/Country MK44 1LQ, United Kingdom

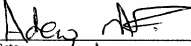
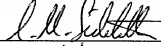

205

Full Name of Inventor <u>S - CC</u>	Family Name <u>SIDESOTTOM</u>	First Given Name <u>Christopher</u>	Second Given Name <u>Michael</u>
Residence & Citizenship	City <u>Bedford</u> <u>GBX</u>	State or Foreign Country United Kingdom	Country of Citizenship United Kingdom
Post Office Address	Post Office Address C/o Unilever Research Colworth, Colworth House, Sharnbrook	City Bedford	State & Zip Code/Country MK44 1LQ, United Kingdom

206

Full Name of Inventor <u>CC - CC</u>	Family Name <u>SMALLWOOD</u>	First Given Name <u>Margaret</u>	Second Given Name <u>Felicia</u>
Residence & Citizenship	City <u>York</u> <u>GBX</u>	State or Foreign Country United Kingdom	Country of Citizenship United Kingdom
Post Office Address	Post Office Address C/o The University of York, Department of Biology, The Plant Laboratory, Heslington	City York	State & Zip Code/Country YO1 5YW, United Kingdom

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signature of Inventor 204 	Signature of Inventor 205 	Signature of Inventor 206 
Date 16/11/99	Date 17/11/99	Date 1 NOVEMBER 1999